Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(6): 748-756
Received: 16-09-2018
Accepted: 18-10-2018

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Screening of lovastatin from higher basidiomycetous fungi from NTCC, forest pathology discipline, Dehradun, India

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Abstract
A study was carried out to screen lovastatin producing ability in Higher Basidiomycetous Fungi isolates from India. For this an extended screening was performed for lovastatin production in Potato Dextrose medium (solid and liquid medium), among a total of 40 basidiomycetous isolates which were obtained from NTCC, Forest Pathology Discipline, Forest Protection Division, Dehradun, Uttarakhand, India). Lovastatin production was evaluated by the disc diffusion method and agar well diffusion method. During the bioassay experiment with *Neurospora crassa* 159 only six isolates were found to be lovastatin producers viz., *Pleurotus ostreatus (NTCC 1253)*, *P. sajor-caju (NTCC 1010)*, *P. floridanus (NTCC 1126)*, *P. eous (NTCC 1264)*, *P. eryngii (NTCC 1070)* and *P. cystidiousus (NTCC 1006)* in liquid and solid growth media with centrifugation and the filtration method.

Keywords: Lovastatin, NTCC, *Neurospora*, Higher Basidiomycetous

1. Introduction
The medicinal properties of basidiomycetous fungi are frequently described in ancient cultures and some have been developed into pharmacological and medicinal applications in modern days [1]. Several basidiomycetous fungi can be engineered to produce various bioactive metabolites, and they remain an important source of novel drugs, including cholesterol-lowering and anticancer agents [2]. Cholesterol-lowering statins (HMG Co-A reductase enzyme inhibitors) are a group of pharmaceuticals that are the most recurrently prescribed for minimizing human deaths due to heart disease [3] Atorvastatin (Lipitor, Pfizer), one of the statins available for treatment, had sales of more than $12 billion worldwide [4]. Lovastatin is a natural product which produces commercial statins, via the polyketide pathway. Although Lovastatin has been reported to be produced by various micro-organisms strains of *Aspergillus* and *Monascus* have been used for commercial production [5-6]. Pharmaceutical industries used *A. terreus* for lovastatin production through the fermentation process. *A. terreus* produces terrain, citrinin, citreoviridin, patulin, sulochrin and benzophenone as co-metabolites of lovastatin [7]. These toxic compounds are also synthesized by polyketide pathway and hence compete with lovastatin biosynthesis for intermediates. Lovastatin production by *A. terreus* has certain draw backdrops, such as high production cost, low yield and extra purification process. These procedures are not only more expensive but also they require the use of a large number of solvents, which in turn are toxic, e.g., ethyl acetate, benzene or acetonitrile. Therefore, new and safer potent lovastatin producing microbial strains are needed currently. Lovastatin, originally isolated from *A. terreus* [8] but actinomycete bacteria and various fungi [9-12] including strains of *P. citrinum* [13] and *M. rubber* [14] have been also reported to produce it. But there are only a few reports on Basidiomycetes species as a potential source of lovastatin [15-17] In this context, the present study focuses on the screening of basidiomycetous fungi from NTCC Forest Pathology Discipline, Dehradun.

2. Materials and methods
A. Fungal Cultures
A forty higher Basidiomycetous isolates were screened for their potential to produce lovastatin (Table 1). Fungal cultures were obtained from NTCC, Forest Pathology Discipline, Forest Protection Division, Forest Research Institute, Dehradun. All isolates were maintained on potato dextrose agar (PDA) slants at 4°C. For bioassay, we used *Neurospora crassa* 159 as test microorganisms which were obtained from MTCC, Chandigarh.

B. Cultivation for Lovastatin Production

To produce inoculants, the fungal isolates were incubated on PDA for 7 days. The agar plugs were punched out using a sterile 6-mm stainless steel cork borer and were transferred onto sterile Petri dishes containing PDA. The inoculated plates were incubated for 7 days at 28°C.

C. Preparation of Fungal Extracts

After incubation, the fungal mycelial discs from duplicate dishes were obtained in agar plugs punched using a sterile 6-mm stainless steel cork borer and transferred to an Eppendorf tube. One millilitre of ethyl acetate was added for lovastatin extraction at 50°C (15 min, with vortex agitation at 2 min intervals). The lovastatin extracts were recovered by centrifugation (2800 × g, 5 min) [18] to determine lovastatin in fungal extracts, pH of extracts was adjusted to 3 using concentrated HCl to convert to a β-hydroxy acid form of lovastatin.

D. Bioassay of Lovastatin

The disc diffusion method [18-19] and agar well diffusion method[23] was used as a screening test for lovastatin activity in the fungal extracts. The fungal extracts were tested against standard test organisms, *Neurospora crassa*. In the bioassay method, a clear zone of inhibition (ZOI) around the test organisms is observed and the diameter of the ZOI is proportional to the concentration of the lovastatin in the samples [18-19]. Test organism was grown for 10 days on PDA slants at 28°C; spores were harvested with 0.85% sterile saline containing 0.2% Tween-80. A hundred microliters of the test organisms were inoculated to a 70-mm-diameter sterilized Petri plate containing PDA medium. For disc diffusion method 6mm-diameter paper discs saturated with 50 µL of the fungal extracts were placed on the surface of *N. crassa*–inoculated plates and for agar well diffusion, wells were made using a sterile cork borer of 6mm diameter and 50 µL of the fungal extract was loaded into wells with the help of micro-pipette. Ethyl acetate was used as a control. All experimental and control plates were incubated at 28°C for 24–48 hours. The bioassay was carried out on the solid media (Potato Dextrose Agar, PDA) and liquid media (Potato Dextrose Broth, PDB) separately with centrifugation, filtration extraction method was used and inhibition zones were recorded [18-19, 23].

Table 1: List of fungal species

S. No.	Fungal Culture	NTCC No.
1	*Pleurotus ostreatus* (Jacq.) P. Kumm.	1253
2	*Pleurotus sajor-caju* (Fr.)Singer	1010
3	*Pleurotus eous* (Berk.) Sacc.	1264
4	*Pleurotus cystidiosus* O.K. Mill	1006
5	*Pleurotus floridanus* Singer	1126
6	*Pleurotus eryngii* (DC.) Quel.	1070
7	*Ganoderma lucidum* (Curtis) P. Karst	1156
8	*Ganoderma applanatum* (Pers.) Pat.	1158
9	*Lenzites trabea* (Pers.) Fr.	90
10	*Oligosporus placentas* (Fr.) Gilb & Ryvarden	276
11	*Schizophyllum commune* Fr.	439
12	*Calocybe indica* Purkay. & A. Chandra	1269
13	*Flavadon flavus*(Klotzsch) Ryvarden	694
14	*Phomopsis rojana* Gaja	1015
15	*Phomopsis spp.*	853
16	*Trametes lactinea* (Berk.)Sacc.	793
17	*Fomitopsis insularis*(Murrill) Imazeki	782
18	*Polyporus dichorus* Fr.	654
19	*Polystictus abientinus* Fr.	561
20	*Irpex flavus* Klotzsch	694
21	*Fomes annosus* (Fr.) Cooke	800
22	*Lenzites betulina* (L.) Fr.	81
23	*Hymenochaete rubiginosa* (Dicks.) Lev.	71
24	*Lenzites striata* Fr.	289
25	*Stereum nitidulum* Berk & M. A. Curtis	214
26	*Phellinus rimosus* (Berk.) Pilat	279
27	*Stereum hirsutum* (Willd) Pers.	368
28	*Trametes hirsuta* (Wulfen) Lloyd	390
29	*Irpex lacteus* (Fr.) Fr.	819
30	*Pycnoporus sanguineus* (L.) Murrill	1272
31	*Heterobasidion annosum* (Fr.) Bref.	1173
32	*Polyporus durus* (Timm) Kreisel	115
33	*Phellinus pachyphloeus* (Pat.) Pat.	358
34	*Phellinus gilvus* (Lloyd) S. Ahmad	648
35	*Phellinus linteus* (Berk & M. A. Curtis)	1252
36	*Trametes versicolor* (L.) Lloyd	1276
37	*Trametes hirsuta* (Wulfen) Lloyd	1265
38	*Laetiporous sulphureus* (Bull.) Murrill	1274
39	*Fomes fermentarius* (L.) Fr.	982
40	*Phellinus allardii* (Bres.) Ryvarden	813

3. Results

A total of 40 fungal isolates obtained from NTCC, Dehradun, India *from Basidiomycetous* sp. were screened for HMG CoA reductase inhibitor activity. All fungal cultures were grown under favourable conditions. The disc diffusion and well diffusion method were used for screening of potential lovastatin producing isolates. Ethyl acetate was used as the control and it did not show any inhibition zone against *Neurospora crassa* lawn culture. As a result of screening, Thirty-four of the screened fungal isolates showed no growth in lovastatin-screening medium but all *Pleurotus* spp. shows positive result viz, *Pleurotus ostreatus (NTCC 1253), P. sajar-caju (NTCC 1010), P. floridanus (NTCC 1126), P. eous (NTCC 1264), P. eryngii (NTCC 1070)* and *P. cystidiousus (NTCC 1006)* (Fig. 1-8). Zones of inhibition of different fungus are shown in table 2.

Observation of different experiments conducted during the course of the study is compiled and detailed in this section. Experiment-wise results/ observation are as follow-

Screening of fungal species for lovastatin production

Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA and Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDA (Table 2).

- In the above experiment assessing *P. ostreatus, P. sajar-caju, P. floridanus, P. eous, P. eryngii* and *P. cystidiosus* on PDA it was found that in agar well diffusion method no clear zone was observed.
- Maximum inhibition was found in *P. cystidious* followed by *P. eryngii, P. floridanus, P. sajor-caju and P. ostreatus.* No clear zone was found in *P. eous (filtration extraction method grown on PDA).*
- In the above experiment assessing *P. ostreatus, P. sajor-caju, P. floridanus, P. eous, P. eryngii* and *P. cystidiosus* on PDA it was found that in agar well diffusion method no clear zone was observed.

Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method) of different fungal species grown on PDB and Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (filtration extraction method) of different fungal species grown on PDB (Table 2).

- In contrast, the culture grown on PDB four species showed a clear zone in disc diffusion method.
- *P. ostreatus* did not show clear zone were grown on PDB and assayed using disc diffusion method.
- Clear zone showing inhibition in test fungi for most of the fungal species were hired when grown on PDA as compared to PDB.
- *P. sajor-caju* and *P. cystidiosus* were the potential lovastatins producing species showing high inhibition zone in different methods.
- From the above experiment, it was also concluded that irrespective of culture medium and method of detection *P. sajor-caju* and *P. cystidiosus* showed a clear zone.
- Lovastatin production in *P. ostreatus* was detected only when grown on PDA.
- Lovastatin production in *P. eous* was detected on both culture mediums only through centrifugation extraction method.
- *P. eryngii* was better-detected centrifugation extraction method when grown on PDA.
- Detection of lovastatin in *P. floridanus* was better using filtration extraction method. The best-suited method for *P. floridanus* was PDA grown cultures with filtration extraction method and its detection with disc diffusion method.

The major findings of the study were:

1. The liquid and solid media extracts of *Pleurotus* spp. were assayed separately. According to results, it was concluded that in comparison to the liquid medium, the solid medium was more able to produce lovastatin.
2. According to lovastatin extraction techniques (centrifugation and filtration) both the extraction methods gave comparable results.
3. Maximum results for lovastatin production were found through a disc diffusion method.

Table 2: Inhibition of *Neurospora crassa* (test fungus) lawn culture by mycelial extract (centrifugation extraction method and filtration method) of different fungal species grown on PDA and PDB

S. No.	Fungal species	Zone of Inhibition (mm) Centrifugation extraction method (PDA)		Zone of Inhibition (mm) Filtration extraction method (PDA)		Zone of Inhibition (mm) Centrifugation extraction method (PDB)		Zone of Inhibition (mm) filtration extraction method (PDB)	
		Disc Diffusion method	Agar Well Diffusion method	Disc Diffusion method	Agar Well Diffusion method	Disc Diffusion method	Agar Well Diffusion method	Disc Diffusion method	Agar Well Diffusion method
1.	*Pleurotus ostreatus*	6.8	ND	10.8	ND	ND	ND	ND	ND
2.	*Pleurotus sajor-caju*	3.2	ND	6.3	ND	6.5	ND	3.2	ND
3.	*Pleurotus floridanus*	2.6	ND	10.2	ND	ND	ND	3.4	ND
4.	*Pleurotus eous*	3.8	ND	ND	ND	3.0	ND	ND	ND
5.	*Pleurotus eryngii*	11.8	ND	11.2	ND	ND	ND	2.0	ND
6.	*Pleurotus cystidiosus*	3.5	ND	13.4	ND	4.4	ND	13.2	ND
7.	Control	ND	ND	ND	ND	ND	ND	ND	ND

Each value is the average of three replications ND- Not detected

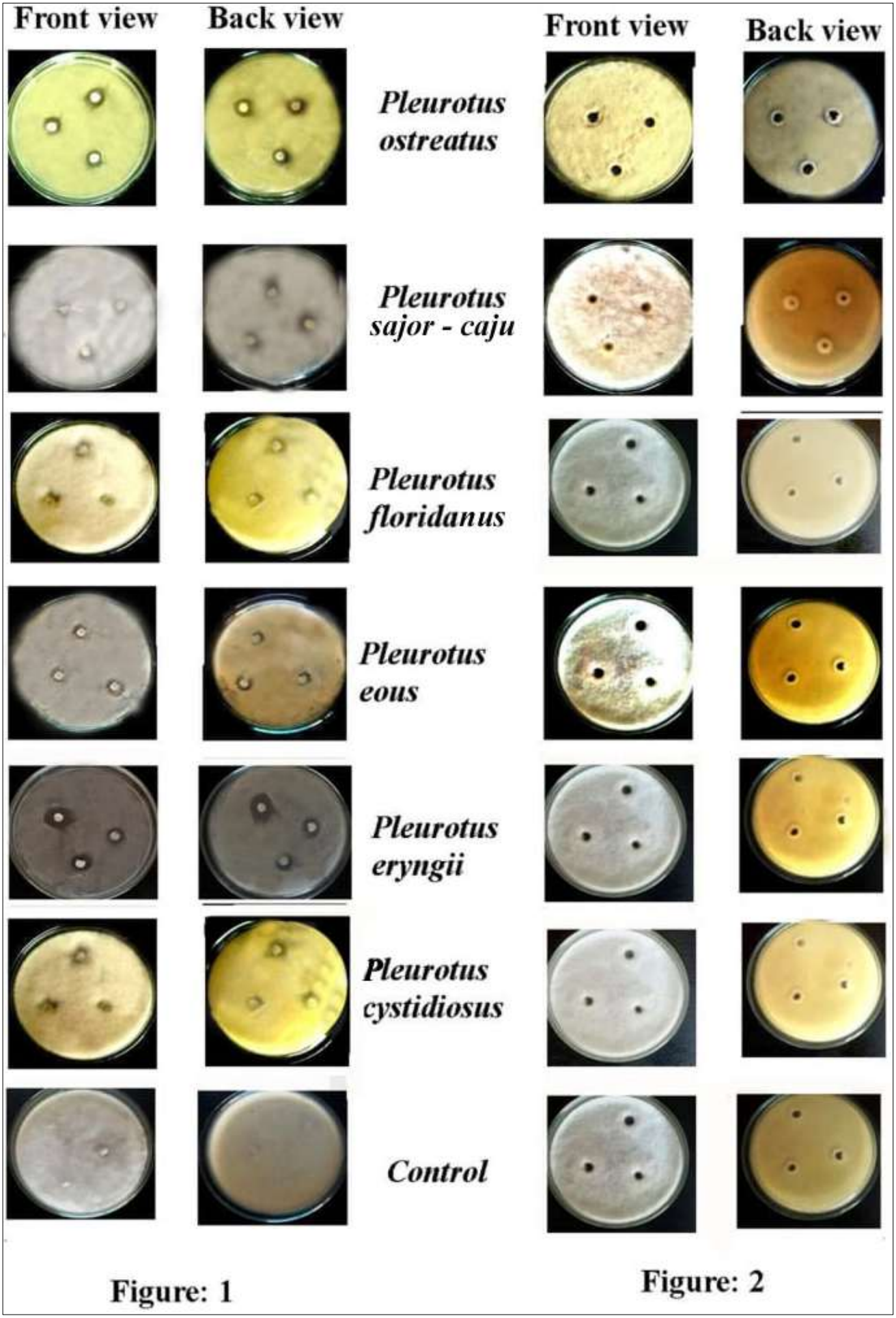

Fig 1, 2: Lawn culture inhibition of *Neurospora crassa* (test fungus) employing a disc diffusion method and agar well diffusion method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDA.

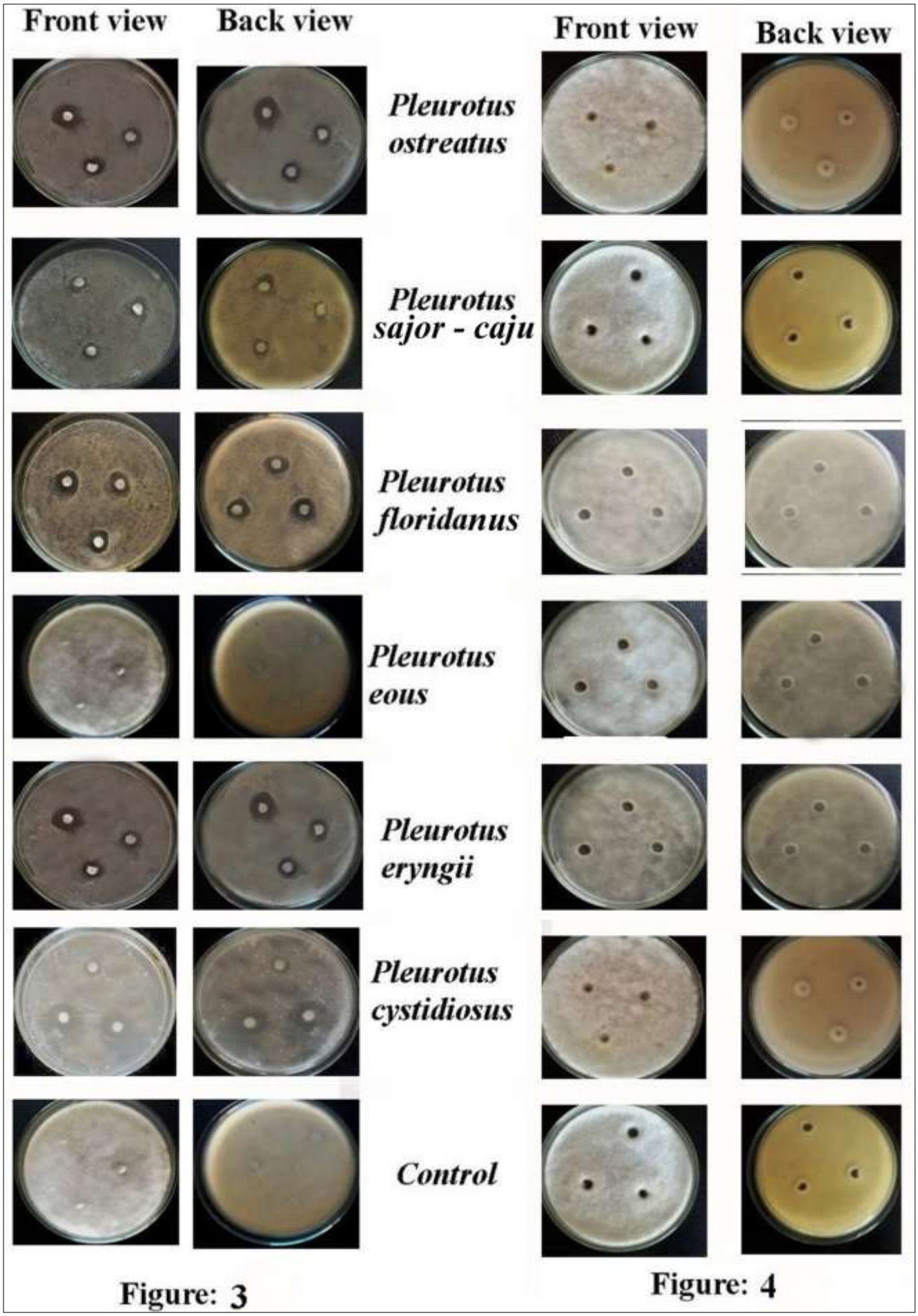

Fig 3, 4: Lawn culture inhibition of *Neurospora crassa* (test fungus) employing disc diffusion method and agar well diffusion method by mycelial extract (filtration extraction method) of different fungal species grown on PDA

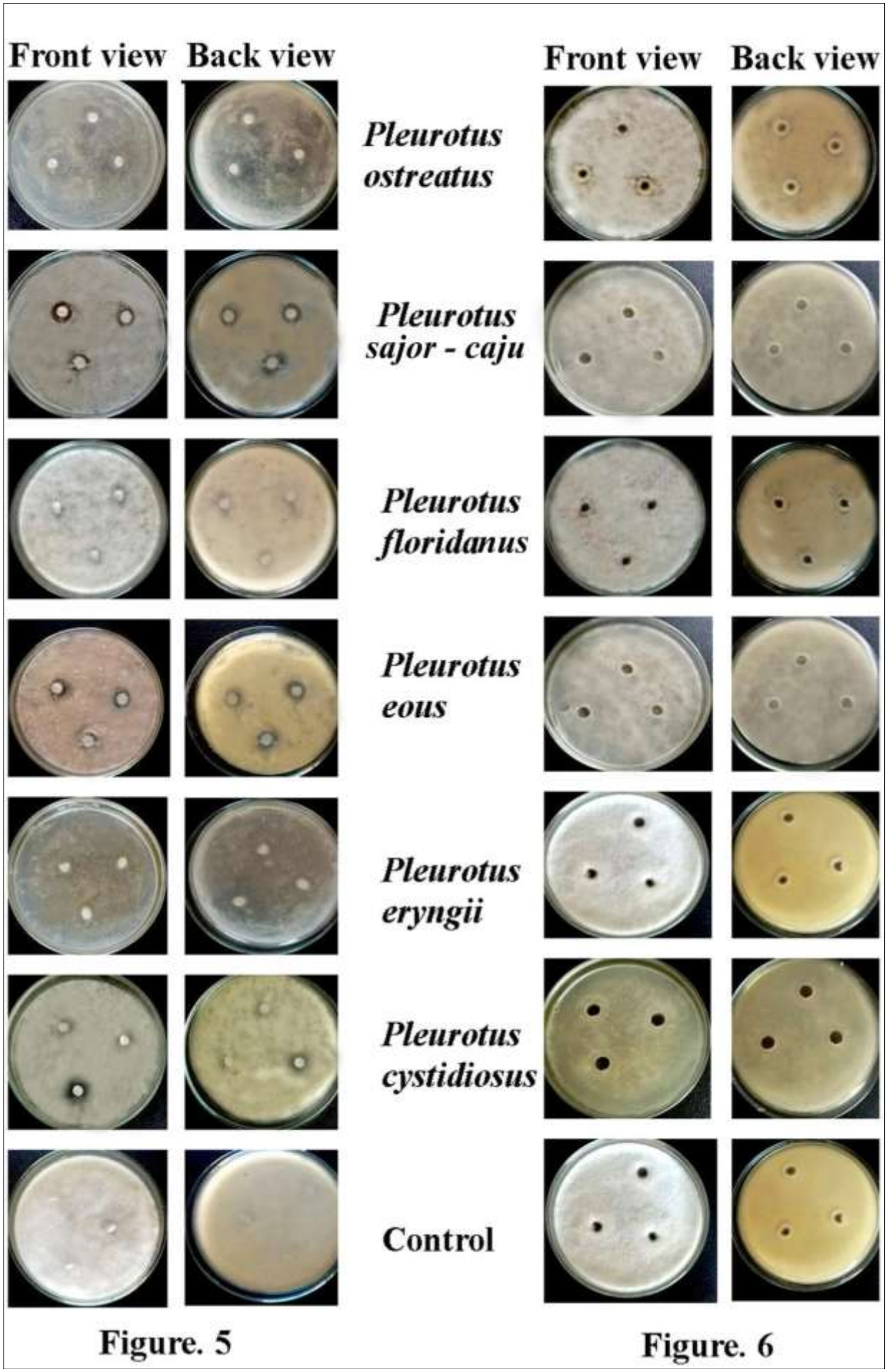

Fig 5, 6: Lawn culture inhibition of *Neurospora crassa* (test fungus) employing disc diffusion method and agar well diffusion method by mycelial extract (centrifugation extraction method) of different fungal species grown on PDB

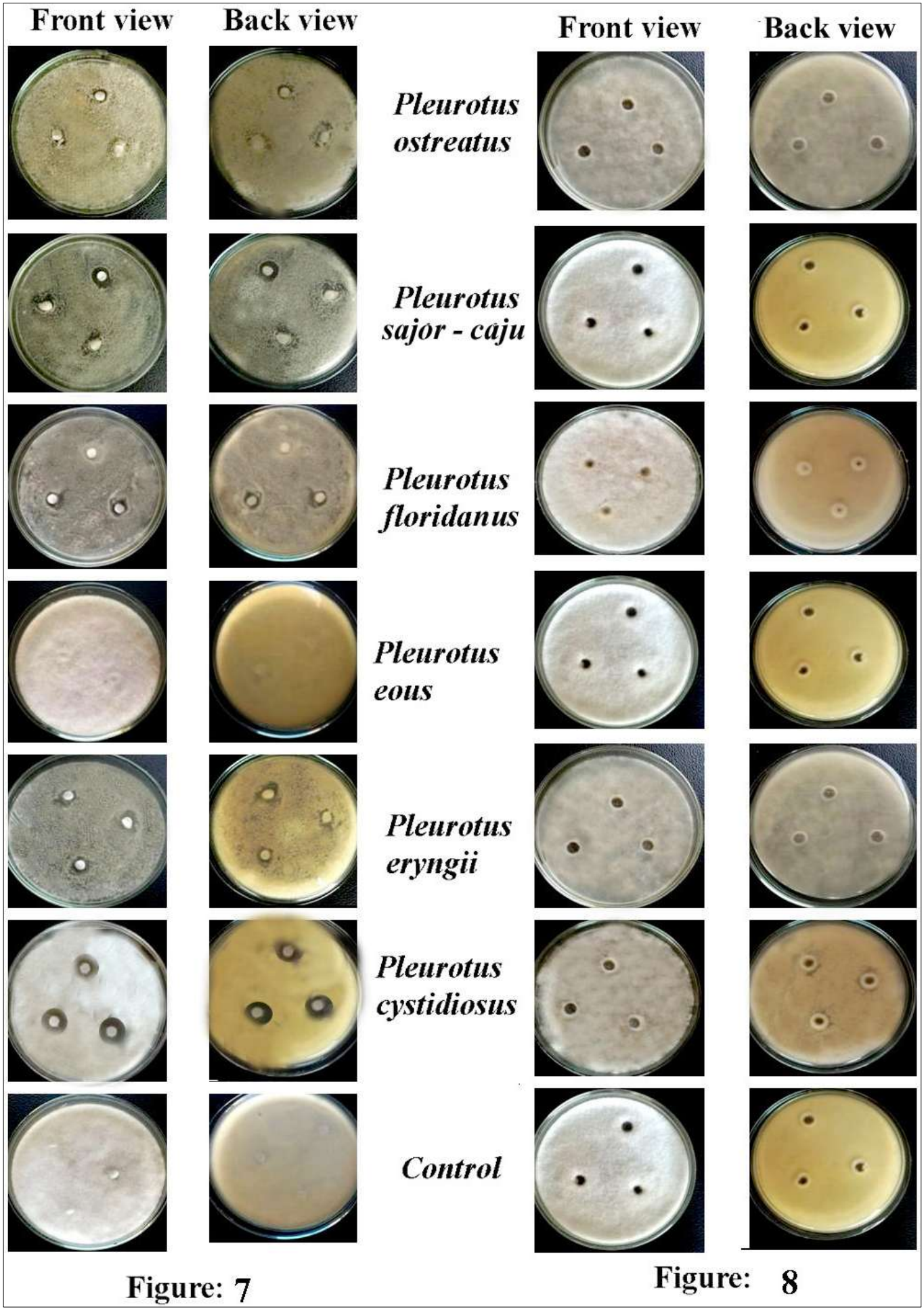

Fig 7, 8: Lawn culture inhibition of *Neurospora crassa* (test fungus) employing disc diffusion method and agar well diffusion method by mycelial extract (filtration extraction method) of different fungal species grown on PDB.

4. Discussion and Conclusion

To find new and capable potent lovastatin producing microbial strains has been an attractive focus for a lot of researchers. Researcher screened 380 fungal strains of 50 different genera and 143 species. They showed that a strain of *Aspergillus terreus* (up to 100 mg/L) and also *Paecilomyces varioti* and *Pythium ultimum* have the ability to produce lovastatin. They also reported lovastatin production in a concentration 1–4.5 mg/L for *Aspergillus flavus, A. niger, A. repens, A. versicolor, Penicillium variable, Pleospora herbarum,* and *Trichoderma viridae*[15]. Another researcher screened 25 fungal species belonging to 14 genera isolated from Egyptian soils for mevinolin production. The results show that *Aspergillus oryzae, A. terreus, Doratomyces stemonitis, P. varioti, Penicillium citrinum, Penicillium chrysogenum, Scopolariopsis brevicaulis,* and *Trichoderma viridae* have the ability to produce lovastatin. *Aspergillus terreus* was the best lovastatin producer (84 mg/L) introduced in his article[11]. Similarly, researcher from Persian Type Culture Collection, screened 110 strains of 22 genera and 50 species and they reported *Aspergillus terreus* (55 mg/L), *Penicillium funiculosom* (19.3 mg/L), *A. umbrosus* (14.1 mg/L), *A. flavus* (9.0 mg/L), *A. parasiticus* (4.5 mg/L), *A. fischeri* (2.0 mg/L), *Trichoderma viridae* (9.0 mg/L), *T. longibrachiatum* (1.0 mg/L), and *Acremonium chrysogenum* (2.5 mg/L) are lovastatin producers[12]. Some researcher also works on actinomycetes source of statin and they screened a total of 65 morphologically different marine Actinomycetes and reported that among screened strains, only one strain (SS16/4) produced HMG Co-A reductase inhibitor[10]. *Hypocrea* and *Penicillium* genera members are potential lovastatins producing isolates from Las Yungas[24] Researcher form Egypt screened 23 fungal isolates isolated from three different locations. They reported that *Aspergillus terreus* 1 (52.9 mg/L), *A. flavus* (48.4 mg/L), *A. oryzae* (37 mg/L), *A. niger* 1 (29 mg/L), *A. terreus* 2 (15.2 mg/L), a Mycelia Sterilia isolate (15.3 mg/L), *Penicillium spinulosum* (15.8 mg/L), and *P. janthinellum* (10.6 mg/L) are good lovastatin producers[25]. A researcher from Andhra Pradesh, India screened various strains of *A. terreus* cultures isolated from soils of different regions of their state. They reported a higher yield of 360 mg/L of lovastatin by a soil fungal isolate, KSV-SUCP-75 (MTCC-10831).

On the other hand, use of higher basidiomycetous fungal isolates that do not produce mycotoxin may be an easy and nontoxic alternative for lovastatin production. Also, a researcher reported that several species of the genus *Pleurotus* and *Agrocybe aegerita*, *Trametes versicolor*, and *Agaricus bisporus* have the ability to produce lovastatin[15]. Similarly, production of lovastatin by higher Basidiomycetes mushrooms, particularly *Pleurotus species*, was reported by different research groups[16, 27-28]. The lovastatin yield is low in the mycelium compared with fruiting bodies of *Pleurotus*[29] High lovastatin contents in the mycelium of *Cordyceps sinensis* and *Agaricus blazei* and fruiting bodies of *A. bisporus* was reported [17].

A researcher from Turkey carried a study for lovastatin production ability in higher Basidiomycetes mushroom isolates. They screened a total of 136 macro fungi, only six macro fungi were showed positive results. The highest production of lovastatin was obtained from the extracts from *Omphalotus olearius* OBCC 2002 (4 mg/L) and *Pleurotus ostreatus* OBCC 1031 (5.8 mg/L) [30].

In spite of these studies, the literature pertaining to lovastatin production by higher basidiomycetous fungal strains is very limited.

Our results showed that all species of *Pleurotus* e.g. *P. ostreatus, P. sajor-caju, P. floridanus, P. eous, P. eryngii and P. cystidiosus* were able to produce inhibition zone against bioassay organism. So these *Pleurotus* species could be used for lovastatin production. Results can be indicative of lovastatin production but this has to confirm further through appropriate chemical analysis with sophisticated instruments like HPLC[31], HPTLC[32]etc.

Acknowledgement

The authors are grateful to the Director, Forest Research Institute, Dehradun, for providing necessary research facilities.

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